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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: PP01651.102; 2302-1651

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/089367

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/IB00/01440	28 September 2000	29 September 1999

TITLE OF INVENTION: MUCOSAL DTPa VACCINES

APPLICANT(S) FOR DO/EO/US: Rino Rappuoli, Mariagrazia Pizza

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is attached hereto (required only if not communicated by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [X] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information: Copy of PCT Request and Fee Calculation Sheet, Copy of Search Report, Copy of Chapter II Demand, Copy of Written Opinion, Copy of International Preliminary Examination Report.

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/089367	PCT/IB00/01440	PP01651.102; 2302-1651

21. ☐ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	34 - 20 =	14	x **$18.00**	$ 252.00	
Independent claims	2 - 3 =	0	x **$84.00**	$ -0-	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$ -0-	
TOTAL OF ABOVE CALCULATIONS =				$1,272.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$	
SUBTOTAL =				$1,272.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$1,272.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$1,272.00	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $ 1,272.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-1648. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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REGISTRATION NUMBER

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MUCOSAL DTPa VACCINES

All documents cited herein are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

This application relates to mucosal DTP vaccines, especially intranasal vaccines.

BACKGROUND TO THE INVENTION

Bordetella pertussis is the causative agent of whooping cough. A highly effective inactivated whole cell vaccine has been available since the 1940s but concern over its safety, due to the presence of toxic cellular components, has limited its uptake [1]. Acellular pertussis vaccines (Pa) comprising a small number of defined *B.pertussis* antigens have therefore been produced, and have been approved for use in humans [2].

Pertussis vaccines are usually administered intramuscularly to children in the form of a trivalent DTP combination (diphtheria, tetanus, pertussis) on alum adjuvant. Intramuscular vaccination is not, however, the ideal route of administration. Mucosal vaccines (oral, intranasal *etc.*) are preferred for two reasons [3]. Firstly, they are easier to administer on a large scale, avoiding the need for specialised equipment and the problems associated with needles. Secondly, they stimulate mucosal immunity, mediated by secretory IgA. As most pathogens enter the body across mucous membranes, mucosal immunity is desirable.

Attempts to make acellular mucosal pertussis vaccines have been described [*e.g.* 4,5,6,7,8,9], but the levels of protection reported were either not compared with conventional vaccine, or did not approach that observed the alum-adjuvanted antigens given parenterally.

There is therefore a need for an effective mucosal DTP combination vaccine.

DISCLOSURE OF THE INVENTION

The invention provides a mucosal DTPa vaccine comprising (a) a diphtheria antigen (D), a tetanus antigen (T), an acellular pertussis antigen (Pa), and (b) a detoxified form of either cholera toxin (CT) or *E.coli* heat labile toxin (LT).

The detoxified form of cholera toxin (CT) or *E.coli* heat labile toxin (LT) acts as a mucosal adjuvant [10]. CT and LT are homologous and are typically interchangeable.

Detoxification of the CT or LT may be by chemical or, preferably, by genetic means. Suitable examples include LT having a lysine residue at amino acid 63 ['LT-K63' – ref. 11], and LT having an arginine residue at amino acid 72 ['LT-R72' – ref. 12], both of which have been found to enhance antigen-specific serum IgG, sIgA, and local and systemic T cell responses to DTPa. LT-K63 is preferred, as this has been found in a reliable animal model of *B.pertussis* infection to result in a high level of protection, equivalent to that generated with a parenterally-delivered DTPa vaccine formulated with alum. Other suitable mutants include LT with a tyrosine at residue 63 ['Y63' – ref. 13] and the various mutants disclosed in reference 14, namely D53, K97, K104 and S106, as well as combinations thereof (*e.g.* LT with both a D53 and a K63 mutation).

The mucosal vaccine of the invention is preferably an intranasal vaccine. In such an embodiment, it is preferably adapted for intranasal administration, such as by nasal spray, nasal drops, gel or powder [*e.g.* 15].

The acellular pertussis antigen preferably comprises pertussis holotoxin (PT) and filamentous haemagglutinin (FHA). It may further comprise pertactin and, optionally, agglutinogens 2 and 3 [16, 17].

PT is a toxic protein and, when present in the pertussis antigen, it is preferably detoxified. Detoxification may be by chemical and/or genetic means. A preferred detoxified mutant is the 9K/129G double mutant [2], referred to herein as 'rPT'.

The diphtheria antigen (D) is preferably a diphtheria toxoid, more preferably the CRM197 mutant [10]. The tetanus antigen (T) is preferably a tetanus toxoid [18].

Non-DTP antigens, preferably ones that do not diminish the immune response against the DTP components, may also be included [*e.g.* ref. 19, which includes a HBV antigen, and ref. 20].

The invention also provides a method of raising an immune response in a patient, comprising administering to a patient a vaccine according to the invention. The immune response is preferably protective against whooping cough, diphtheria and tetanus. The patient is preferably a child.

The method may raise a booster response, in a patient that has already been primed against *B.pertussis*. The primer vaccination may have been by a mucosal or parenteral route.

The invention also provides the use of a detoxified mutant of cholera toxin (CT) or *E.coli* heat labile toxin (LT) in the manufacture of an intranasal medicament for vaccinating a patient

against whooping cough, diphtheria and tetanus, or for boosting an primer immune response previously raised against *B.pertussis*.

The invention also provides an immunogenic composition comprising (a) a diphtheria antigen (D), a tetanus antigen (T), an acellular pertussis antigen (Pa), and (b) a detoxified form of either cholera toxin (CT) or *E.coli* heat labile toxin (LT).

It will be appreciated that references in the above text to particular proteins (*e.g.* pertactin, PT, *etc.*) encompass their allelic variants and functional mutants. They also encompass proteins having significant sequence identity to the wild-type proteins. The degree of identity is preferably greater than 50% (*e.g.* 65%, 80%, 90%, or more) calculated using, for instance, the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters *gap open penalty=12* and *gap extension penalty=1*. Immunogenic fragments of these proteins may also be used, as may longer proteins incorporating the proteins, variants or fragments (*e.g.* fusion proteins). In all cases, however, the protein (whether wild-type, variant, mutant, fragment or fusion) will substantially retain the wild-type immunogenicity.

The proteins can, of course, be prepared by various means (*e.g.* recombinant expression, purification from cell culture, chemical synthesis *etc.*) and in various forms (*e.g.* native, fusions *etc.*). They are preferably prepared in substantially pure or isolated form (*ie.* substantially free from other bacterial or host cell proteins with which they are normally associated in nature)

The vaccines of the invention may comprise nucleic acid for 'genetic immunisation' [*e.g.* 21]. The nucleic acid will encode a protein component of the vaccine and may replace individual protein components, or may supplement them. As an example, the vaccine may comprise DNA that encodes a tetanus toxin.

Vaccines according to the invention will typically be prophylactic (*ie.* to prevent infection), but may also be therapeutic (*ie.* to treat disease after infection).

The vaccines of the invention will, in addition to components (a) and (b), typically comprise 'pharmaceutically acceptable carriers', which include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid

copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. The vaccines may also contain diluents, such as water, saline, glycerol, *etc.* Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of antigen, as well as any other of the above-mentioned components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows T-cell responses in spleen to an intranasal Pa vaccine adjuvanted with LT-K63. The T cell stimulus used in the assay was: PT (filled), FHA (diagonal shading), or *B.pertussis* bacteria (horizontal lines). Pa vaccine (FHA+rPT) was delivered with or without LT-K63 adjuvant, with or without light halothane anaesthesia. PBS was a control. **Figure 2** shows similar data for LT-R72 adjuvant, in (A) spleen (B) thoracic lymph node (C) superficial cervical lymph node. PMA/CD3 (no shading) was used as a positive control. **Figure 3** shows antibody responses for the same vaccines – 3A shows results using LT-K63 adjuvant, and 3B shows results using LT-R72 adjuvant. Filled bars show anti-PT responses; empty bars show anti-FHA responses.

Figure 4 shows the effect of toxin dose on the adjuvant effect of the mutant LT adjuvants.

Figure 5 shows the kinetics of *B.pertussis* clearance after immunisation with the same vaccines as Figures 1 & 2 – 5A shows results using LT-K63, and 5B shows results using LT-R72. Results are the mean viable *B.pertussis* for individual lungs from four mice per time point per experimental group.

Figure 6 shows the IgA and IgG responses against the five antigens in a DTPa vaccine, comparing (i) alum adjuvant and intramuscular administration (empty bars) and (ii) LT-K63 adjuvant and intranasal administration (filled bars). **Figure 7** compares T cell responses for the same vaccines, and **Figure 8** shows the clearance kinetics.

Figure 9 shows T-cell proliferation (measured as ^{3}H-CPM) against the D (bottom), T (middle) and Pa (top) components of DTPa vaccines administered using 5 different prime and boost regimens. The T-cell cytokine responses against the Pa component (**Figure 10**), the D component (**Figure 11**) and the T component (**Figure 12**) are also shown. **Figure 13** shows serum IgG (top) and lung homogenate IgA (bottom) titres (log_{10}) in response to the five defined antigens in the DTPa mixture. **Figure 14** shows the functionally important anti-DT neutralising antibodies. **Figure 15** shows clearance kinetics for the five regimens.

Where calculated, statistical significance (Student's *t* test) *versus* Pa alone is indicated by either * ($P<0.05$) or ** ($P<0.01$).

MODES FOR CARRYING OUT THE INVENTION

Background materials & methods

Mice used in the following examples were female BALB/c mice, 6-8 weeks old, from Harlan UK and were housed according to the regulations of the Irish Department of Health.

T-cell responses Mice were immunised at 0 and 4 weeks. At 6 weeks, spleen, superior cervical lymph nodes and posterior mediastinal (thoracic) lymph nodes were removed and immune responses were evaluated. Spleen cells from individual mice or pooled lymph node cells ($2x10^6$ cells/ml) from naïve or immunised mice were cultured in triplicate in 8% FCS supplemented RPMI at 37°C with heat-killed (80°C for 30 minutes) *B.pertussis* bacteria (10^6 or 10^7 cells/ml), heat inactivated rPT (1-5μg/ml), or FHA (1-5μg/ml). Phorbal myristate acetate (PMA) + anti-mouse CD3 was used as a positive control; medium only was used as a negative control. In experiments using DTPa, responses were also tested against PRN, TT, or CRM197 (1-5μg/ml). Supernatants were removed after 72 hours and the concentration of IFN-γ (indicative of Th1 response) and IL-4 & IL-5 (both indicative of Th2 response) were determined by immunoassay as described in reference 22. T-cell proliferation was assessed after 4 days of culture by ^{3}H-thymidine uptake, also as described in reference 22. Results are expressed as mean counts per minute or mean cytokine concentration for the optimum concentration of antigen in assays performed in triplicate on individual spleen cells or pooled lymph node cells from four to five mice.

Antibody assays Levels of antigen-specific IgG in the serum of control and immunised mice were determined by ELISA. Purified antigens (FHA, PT, TT and DT; 1.0μg/ml) were used to coat the ELISA plates. The plates were blocked with milk protein, then serially diluted serum samples were added, the bound antibody was detected by anti-mouse IgG (Fc-specific) alkaline-phosphatase conjugate. Antigen-specific IgA in lungs was detected by ELISA. Lungs were homogenised in 8% FCS supplemented RPMI containing 0.1mM PMSF protease inhibitor. ELISA plates were coated with antigen as for the IgG assay and serially diluted lung homogenate was added. Bound antibody was detected with sheep anti-mouse IgA, followed by donkey-anti-sheep IgG alkaline phosphatase conjugate. Results are expressed as end point titres, calculated by regression of the straight part of a curve of optical density versus serum or lung homogenate dilution to a cut-off of 2 standard deviations above background control values for serum or lung homogenates from naïve mice.

1) LT mutants are intranasal adjuvants for Pa

Two Pa vaccines were prepared. The antigen component in each vaccine was FHA (2.5μg/dose) + rPT (5.0μg/dose), with antigens prepared as described in reference 23.

The first vaccine (Figure 1) was adjuvanted with LT-K63 (10μg/dose), whereas the second vaccine (Figure 2) was adjuvanted with LT-R72 (1μg/dose). A control vaccine consisted of FHA + rPT only. The adjuvants were prepared as described in references 24 and 25.

Mice were immunised at 0 and 4 weeks either with the vaccine dose resuspended in 25μl and applied to the external nares with a micropipette or, following light halothane anesthesia, with the vaccine dose resuspended in 50μl and applied to the external nares with a micropipette. T-cell responses to killed *B.pertussis,* heat-inactivated PT and FHA were measured in spleen and thoracic and cervical lymph nodes at 6 weeks (Figures 1 & 2).

Strong T-cell proliferation and cytokine production was detected for the adjuvanted Pa vaccines. In contrast, spleens and local lymph nodes from mice intranasally immunised with the control failed to generate significant *B.pertussis*-specific T-cell responses. Positive responses to the polyclonal stimulus (PMA + anti-CD3) confirms that these T-cells were capable of responding *in vitro*.

Figure 3 shows that the mutant LT adjuvants also enhanced local and systemic antibody production following intranasal delivery of Pa. Immunisation with the control generated weak and inconsistent anti-PT and anti-FHA serum IgG and lung IgA responses. In contrast, formulation of the same antigens with LT-R72 or LT-K63 resulted in consistently strong

serum IgG and lung IgA specific for PT and FHA and also significantly enhanced IgA responses, especially when the vaccine was administered under anaesthesia.

The presence of the LT mutants thus resulted in better T-cell and antibody responses. They can enhance the protective efficacy of a nasally delivered Pa, and are therefore effective intranasal adjuvants for acellular vaccines.

2) Effect of enzyme activity and toxin dose on adjuvanticity

The cytokine profiles obtained in example 1 that the ADP-ribosylation activity of the toxins plays an important role in the modulation of the immune response. The K63 adjuvant, which is devoid of any toxic enzyme activity, enhanced the production of IL-4, IL-5, and IFN-γ, characteristic of a mixed Th1-Th2 (*i.e.* Th0) profile. In contrast, 1.0μg of the R72 adjuvant, which retains partial toxic enzyme activity, appeared to selectively enhance Th2 cells.

In experiments that directly compared the adjuvanticity of the toxins *in vivo*, BALB/c mice were immunised with Pa formulated with 1 or 10μg of LTK63 or LTR72 as adjuvant, and the resulting immune responses were assessed (Figure 4). Intranasal immunisation with control Pa generated weak T-cell responses, whereas addition of 1μg LTK63 enhanced proliferation, as well as IFN-γ and IL-5 production, by spleen cells and lymph nodes in response to FHA or killed *B.pertussis*. Increasing the dose to 10μg LTK63 resulted in modest further enhancements of proliferation and IFN-γ production. 1.0μg LTR72 selectively augmented Th2 responses, with elevated levels of antigen-induced IL-4 and IL-5 production compared with those observed with Pa alone. Wild-type LT (1.0μg) also selectively enhanced IL-4 and IL-5 production, but the effect was not as dramatic as that observed with LTR72. Furthermore, the mice that received 1.0μg LTR72 had significantly higher anti-FHA and anti-PT IgG and IgA antibody titres than those immunised using LTK63 or wild-type LT (data not shown). Increasing the dose of LTR72 from 1.0 to 10μg resulted in enhancement of IFN-γ levels and lower levels of IL-4 and IL-5.

Thus, the enzyme activity and the dose of the toxin appear to affect the cytokine profile of the antigen-specific T cells induced. The trace amounts of ADP-ribosylating activity present in low doses of LTR72 are sufficient to modulate the cytokine profile to Th2 and act as a potent adjuvant for antibody responses. Conversely, the adjuvant effect of LTK63, which is mediated by the binding effect of the AB complex, is pushed more toward the Th1 subtype. Furthermore, at higher doses of LTR72, the AB binding activity may outweigh the enzyme activity, resulting in enhancement of Th1 as well as Th2 cell induction.

3) Protection against pertussis infection

Vaccine efficacy in human clinical trials has been correlated with the protection of immunised mice in the respiratory challenge model described in reference 22. This model was therefore used to assess intranasally delivered Pa formulated with the LT adjuvants, in order to predict human efficacy.

B.pertussis W28 phase I was grown under agitation conditions at 37°C in Stainer-Scholte liquid medium. Bacteria from a 48 hour culture were resuspended at a concentration of approximately $2x10^{10}$cells/ml in physiological saline containing 1% casein. The challenge inoculum was administered to mice over a period of 15 mins by means of a nebuliser, followed by rest in the chamber for a further 15 mins. Groups of 4 mice were sacrificed at 0, 3, 7, 10 and 14 days, and the number of viable *B.pertussis* in the lungs were assessed. Lungs were removed aseptically from infected mice and homogenised in 1ml sterile physiological saline with 1% casein on ice. Aliquots of 100µl undiluted or serially diluted homogenate from individual lungs were spotted in triplicate onto Bordet-Genou agar plates, and the number of colonies was assessed after 5 days incubation (Figure 5).

The adjuvanted Pa formulations provided levels of protection significantly greater than those achieved with soluble antigens alone. The LT-K63 adjuvant generated marginally better protection than LT-R72. Nasal delivery of Pa with LT-R72 in 25µl (no anaesthetic) gave marginally better protection than the same vaccine in 50µl (with anaesthetic). Neither of these two differences was significant.

The protection levels shown in Figure 5 exceed those previously observed with a conventional parenterally delivered two component Pa (25µg FHA + 25µg chemically-detoxified PT on alum [16,22]). Extrapolation of the correlation curve shows a better potency index, suggesting superior clinical efficacy in humans.

4) DTPa efficacy using LT-K63

Pertussis vaccines are usually administered intramuscularly to children in the form of a trivalent DTP combination on alum adjuvant. To assess the efficacy of intranasal vaccination, a DTPa vaccine was therefore adjuvanted with alum (300µg/dose, 300µl volume) for intramuscular administration, for direct comparison with the LT-K63-adjuvanted intranasal vaccine (10µg adjuvant/dose, 40µl volume). The Pa component of the vaccine included 5µg rPT, 2.5µg FHA and 2.5µg pertactin; the T component was 10µg tetanus toxoid; the D component was 10µg CRM197.

The intranasal vaccine enhanced cellular and humoral immune responses to tetanus and diphtheria as well as pertussis antigens (Figures 6 & 7). The levels of serum IgG using the intranasal vaccine were equivalent to those observed using the intramuscular vaccine, but the mucosal immunisation advantageously enhanced local IgA responses.

Significantly, the protective efficacy of the LT-K63-adjuvanted vaccine matched that of the 'standard' alum-adjuvanted vaccine, although clearance kinetics varied slightly (Figure 8). This is the first disclosure of a mucosally-delivered combined DTPa formulation that is capable of generating a level of protection against *B.pertussis* infection equivalent to that observed with the same antigens adsorbed on alum and administered parenterally.

5) Intramuscular priming and intranasal booster

The DTPa vaccine was also used in a prime-boost experiment.

Two groups of 22 mice were immunised intramuscularly at 0 and 4 weeks with either DTPa on alum, or PBS (control). A further group of 22 mice was immunised intranasally at 0 and 4 weeks with the LT-K63-adjuvanted vaccine. Two further groups of 22 mice were immunised with the intramuscular alum formulation at week 0, and the intranasal formulation (with or without LT-K63 adjuvant) at week 4:

Group	Priming dose	Boosting dose
1	PBS	PBS
2	Intramuscular DTPa (alum)	Intramuscular DTPa (alum)
3	Intramuscular DTPa (alum)	Intramuscular DTPa (PBS)
4	Intramuscular DTPa (alum)	Intranasal DTPa (LT-K63)
5	Intranasal DTPa (LT-K63)	Intranasal DTPa (LT-K63)

Five mice from each group were sacrificed at week 6, and serum, lungs and spleen cells were measured for immune responses. The remaining mice were subjected to the infection model. One mouse from each group on day 0 and four mice from each group on days 3, 7, 10 and 14 were sacrificed, and their CFU-counts were measured from their lungs.

T-cell proliferation (Figure 9) was weak for all groups for spleen cells stimulated with the pertussis antigens *in vitro*. The cells did, however, proliferate in response to the positive control (PMA+CD3). Proliferation responses to tetanus toxoid *in vitro* were significantly stronger in intranasally-boosted mice (after intramuscular priming) when LT-K63 was used as

adjuvant. The strongest *in vitro* proliferation against the diphtheria component was seen in the mice immunised intranasally twice.

Cytokine responses to pertussis antigens (Figure 10) showed both IL-5 and IFN-γ production in all groups, indicating priming of both Th1 and Th2 populations *in vivo*. IL-4 production was limited to groups immunised in the same way both times. Priming and boosting with the intranasal LT-K63 formulation seems to give a stronger Th2 response (higher IL-4 and IL-5) than the groups primed intramuscularly.

Cytokine responses against the diphtheria antigen (Figure 11) were restricted to IL-4 and IL-5, with little or no IFN-γ detected for any group. Intranasal boosting with DTPa thus results in the priming of Th2 cells *in vivo*. The strongest Th2 response was generated from the mice immunised intranasally twice with the LT-K63 adjuvant. In contrast, two intramuscular injections gave no detectable IL-4 or IL-5 responses in the spleen, nor nay IFN-γ.

Cytokine responses against the tetanus antigen (Figure 12) showed the production of IL-4, IL-5 and low levels of IFN-γ in all mice, indicating a mixed Th1/Th2 response. IL-4 and IL-5 levels were, however, significantly higher in groups boosted intranasally with the LT-K63 adjuvant, compared with the non-adjuvanted intranasal booster or the intramuscular booster.

IgG responses against TT, DT and PTN (Figure 13) showed no significant differences in titres between the various groups. Anti-PT and anti-FHA titres were slightly higher in groups primed and boosted with DTPa intramuscularly than in groups boosted intranasally (with or without LT-K63 adjuvant). Anti-FHA IgG were not detected, although this is not in agreement with the results presented above. IgA levels (Figure 13) showed that intramuscular priming and intranasal boosting using the LT-K63 adjuvant generated similar titres for most antigens to intranasal priming and boosting, although anti-PT levels were significantly lower. Intranasal boosting without the LT-K63 adjuvant generated lower IgA levels, particularly for DHA and PTN.

Analysis of functionally important anti-DT neutralising antibodies in murine sera (Figure 14) demonstrated that intramuscular priming and intranasal boosting using LT-K63 resulted in the highest levels.

The protection model showed that similar levels of protection was obtained in the dual-intranasal and dual-intramuscular immunisations. Whilst the kinetics of the clearance curves (Figure 15) vary, *B.pertussis* was effectively cleared in both cases, with CFU counts below 1

(log_{10}) 14 days after challenge. Most adults today have received an intramuscular pertussis vaccination. This is represented by the intramuscular priming in this example. The data show that intranasal boosting with LT-K63 adjuvant is an effective method of vaccination.

This example also shows that LT-K63 is a very effective adjuvant for the delivery of 1X CRM197. Intranasal enhancement against this antigen has been reported using chitosan, although this required three immunisations for modest IgA and T-cell responses. In contrast, LT-K63 was able to induce strong IgG, IgA, IL-4 and IL-5 responses after just two intranasal immunisations. Similar levels of anti-DT neutralising antibodies were also generated as with chitosan.

It will be understood that the invention is described above by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

REFERENCES (the contents of which are incorporated herein in full by reference)

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12. WO98/18298

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19. WO93/24148.

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22. Mills *et al.* (1998) *Infect. Immun.* 66:2594-2602.

23. Podda *et al.* (1991) *Vaccine* 9:741-745.

24. Douce *et al.* (1995) *PNAS USA* 92:1644-1648.

25. Giulani *et al.* (1998) *J. Exp. Med.* 187:1-10.

CLAIMS

1. A mucosal DTPa vaccine comprising:
   (a) a diphtheria antigen, a tetanus antigen, and an acellular pertussis antigen; and
   (b) a detoxified form of either cholera toxin or *E.coli* heat labile toxin.

2. The DTPa vaccine of claim 1, wherein component (b) is LT-K63 or LT-R72.

3. The DTPa vaccine of claim 1 or claim 2, adapted for intranasal administration.

4. The DTPa vaccine of any preceding claim, wherein the acellular pertussis antigen comprises detoxified pertussis holotoxin and filamentous haemagglutinin and, optionally, pertactin.

5. The DTPa vaccine of claim 4, wherein the detoxified pertussis holotoxin is a 9K/129G double mutant.

6. The DTPa vaccine of any preceding claim, wherein the diphtheria antigen is the CRM197 mutant and the tetanus antigen is a tetanus toxoid.

7. The DTPa vaccine of any preceding claim, further comprising a non-DTPa antigen.

8. A method of raising an immune response in a patient, comprising administering to a patient a vaccine according to any preceding claim.

9. The method of claim 8, wherein the patient is a child.

10. The method of claim 8, wherein the vaccine is given as a booster.

11. A composition comprising (a) a diphtheria antigen, a tetanus antigen, and an acellular pertussis antigen and (b) a detoxified form of either cholera toxin or *E.coli* heat labile toxin for use as a vaccine.

12. The use of a detoxified mutant of cholera toxin or *E.coli* heat labile toxin in the manufacture of an intranasal medicament for vaccinating a patient against whooping cough, diphtheria and tetanus.

13. The use of a detoxified mutant of cholera toxin or *E.coli* heat labile toxin in the manufacture of an intranasal medicament for booster vaccination of a patient against whooping cough, diphtheria and tetanus.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(75) Inventors/Applicants *(for US only)*: **RAPPUOLI, Rino** [IT/IT]; Chiron S.p.A., Via Fiorentina, 1, I-53100 Siena (IT). **PIZZA, Mariagrazia** [IT/IT]; Chiron S.p.A., Via Fiorentina, 1, I-53100 Siena (IT).

(74) Agents: **HALLYBONE, Huw, George** et al.; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

(81) Designated States *(national)*: CA, JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *with international search report*

(88) Date of publication of the international search report:
25 October 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/22993 A3

(54) Title: MUCOSAL DTPa VACCINES

(57) Abstract: Mucosal DTPa vaccines, especially intranasal vaccines, comprising (a) a diphtheria antigen, a tetanus antigen and an acellular pertussis antigen, and (b) a detoxified mutant of cholera toxin (CT) or *E.coli* heat labile toxin (LT). Component (b) acts as a mucosal adjuvant. The acellular pertussis antigen preferably comprises pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) and, optionally, pertactin. The mucosally-delivered combined DTPa formulation is capable of generating a level of protection against *B.pertussis* infection equivalent to that observed by alum-adjuvanted parenteral administration.

FIGURE 1

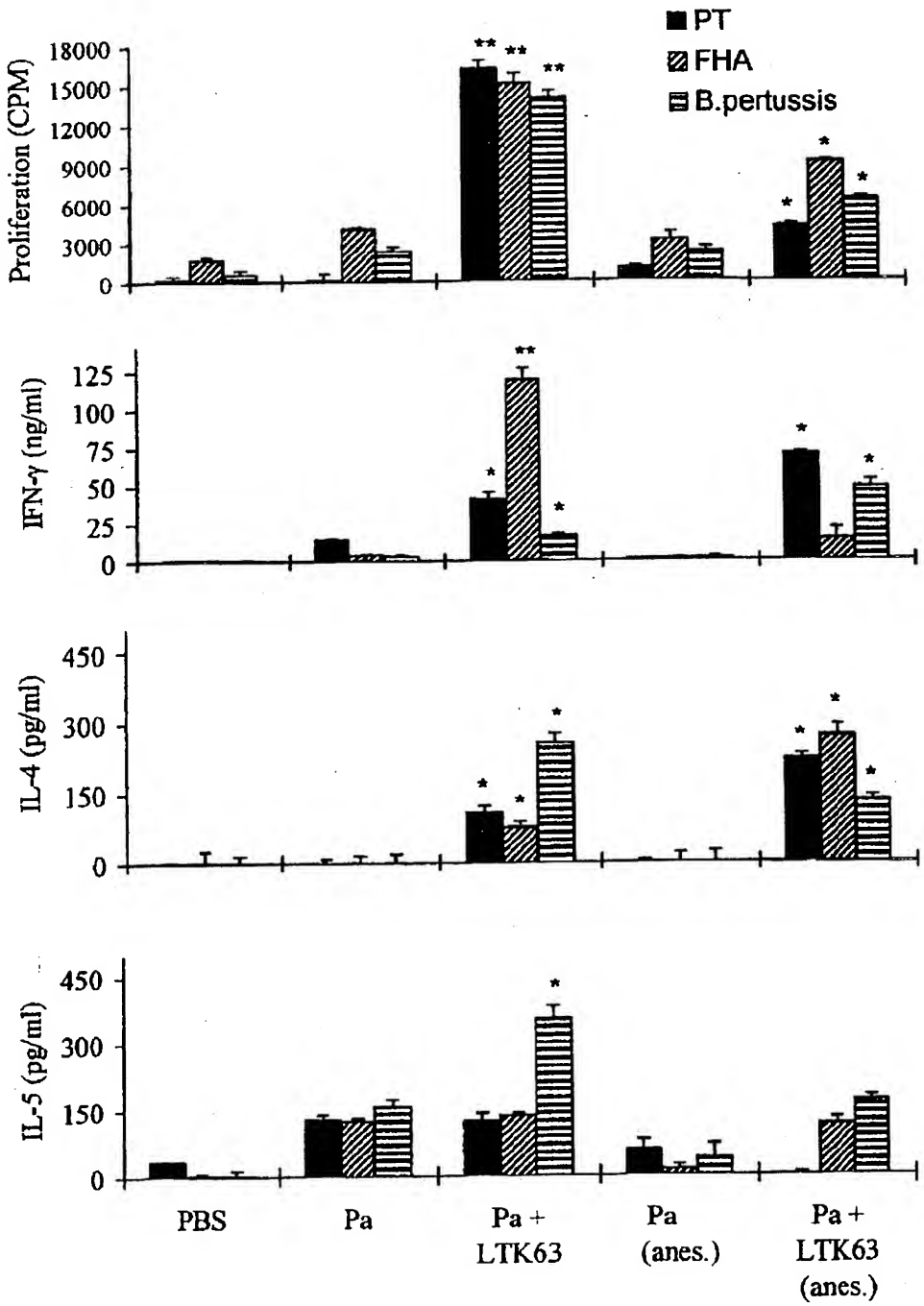

PT
FHA
B.pertussis
Proliferation (CPM)
18000
15000
12000
9000
6000
3000
0
IFN-γ (ng/ml)
125
100
75
50
25
0
IL-4 (pg/ml)
450
300
150
0
IL-5 (pg/ml)
450
300
150
0
PBS
Pa
Pa + LTK63
Pa (anes.)
Pa + LTK63 (anes.)

FIGURE 2A

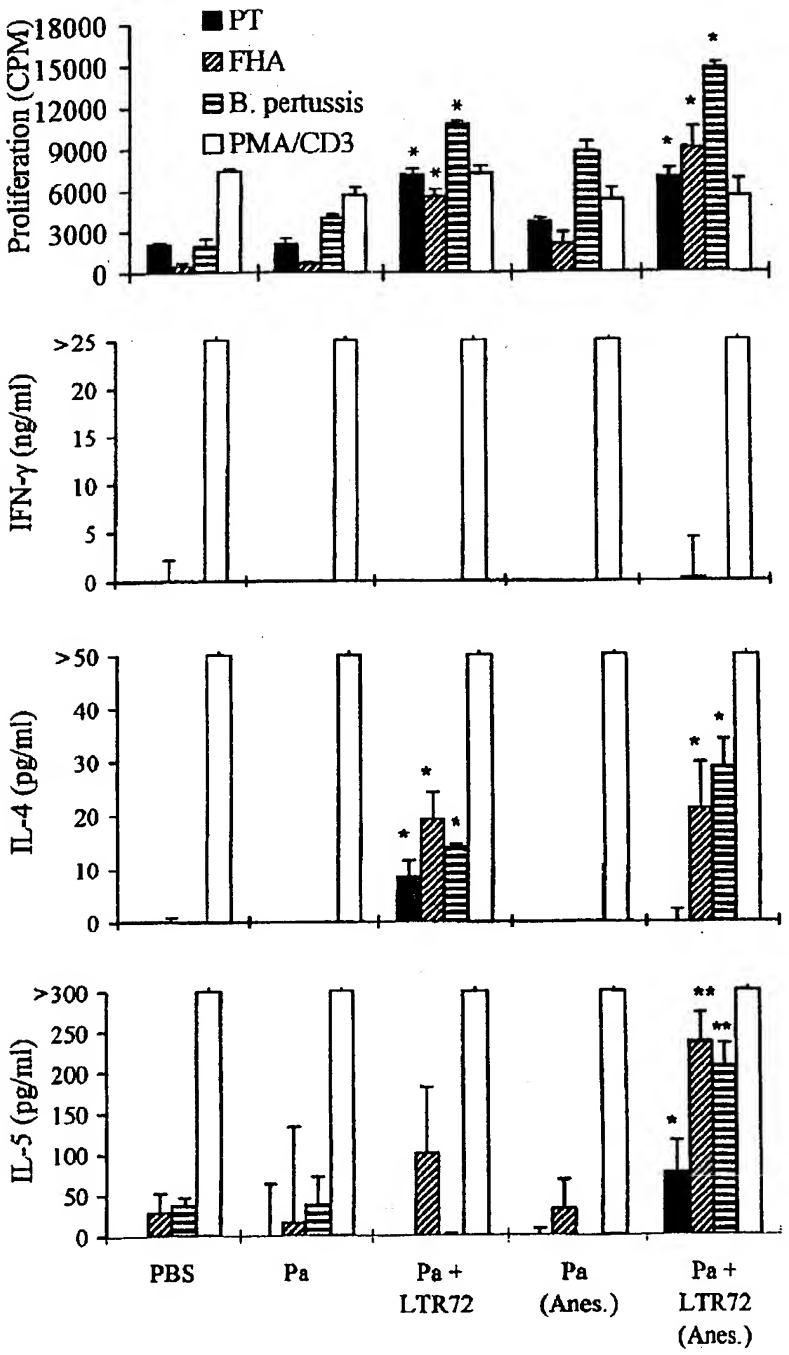

PT
FHA
B. pertussis
PMA/CD3
Proliferation (CPM)
18000
15000
12000
9000
6000
3000
0
IFN-γ (ng/ml)
>25
20
15
10
5
0
IL-4 (pg/ml)
>50
40
30
20
10
0
IL-5 (pg/ml)
>300
250
200
150
100
50
0
PBS
Pa
Pa + LTR72
Pa (Anes.)
Pa + LTR72 (Anes.)

FIGURE 2B

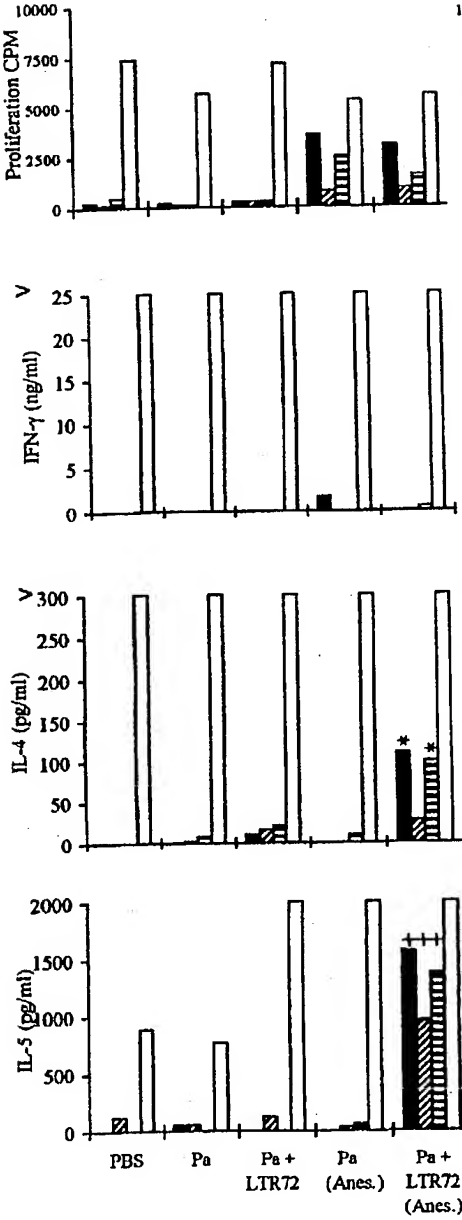


FIGURE 2C

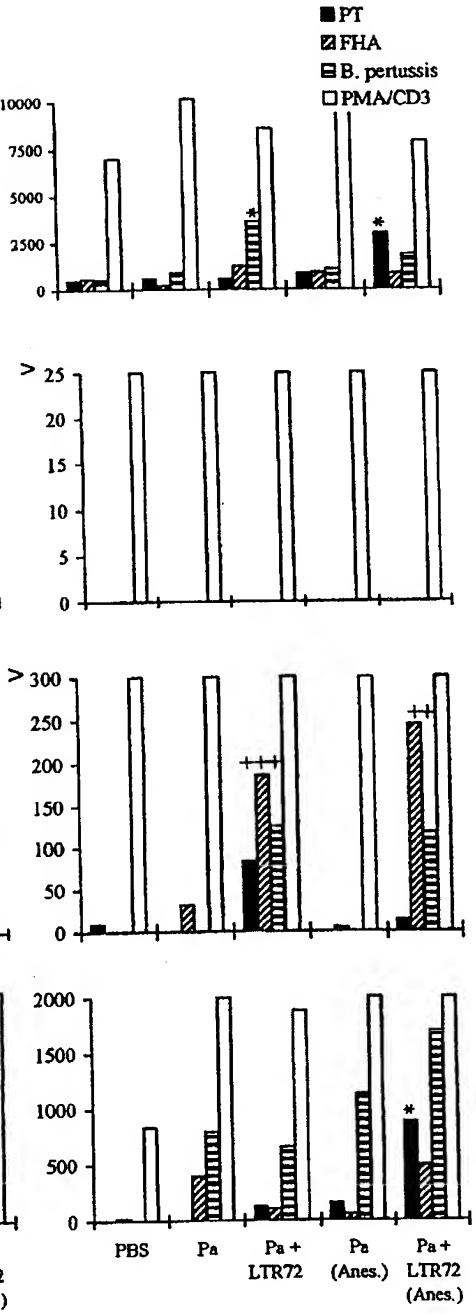


FIGURE 3A

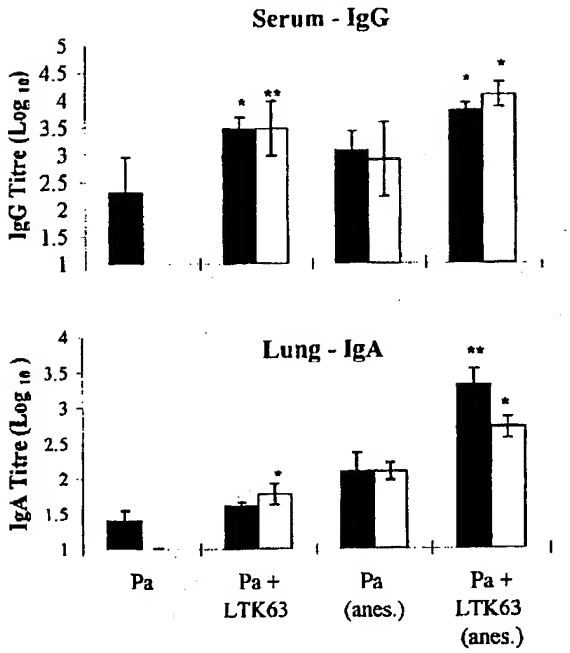


FIGURE 3B

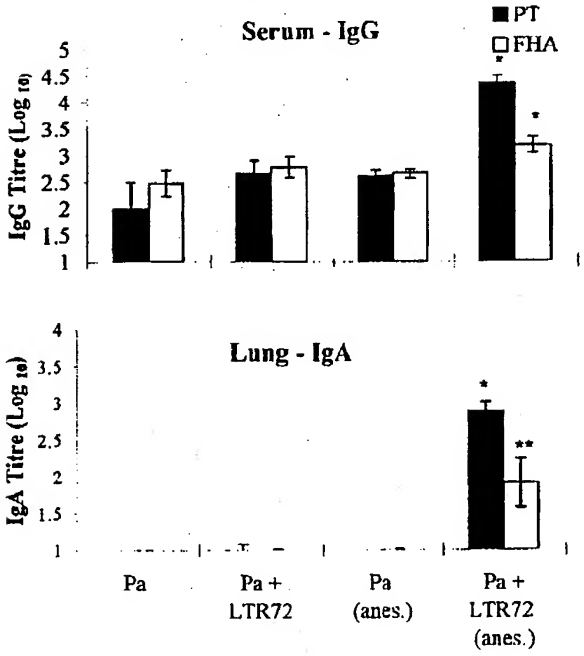


FIGURE 4

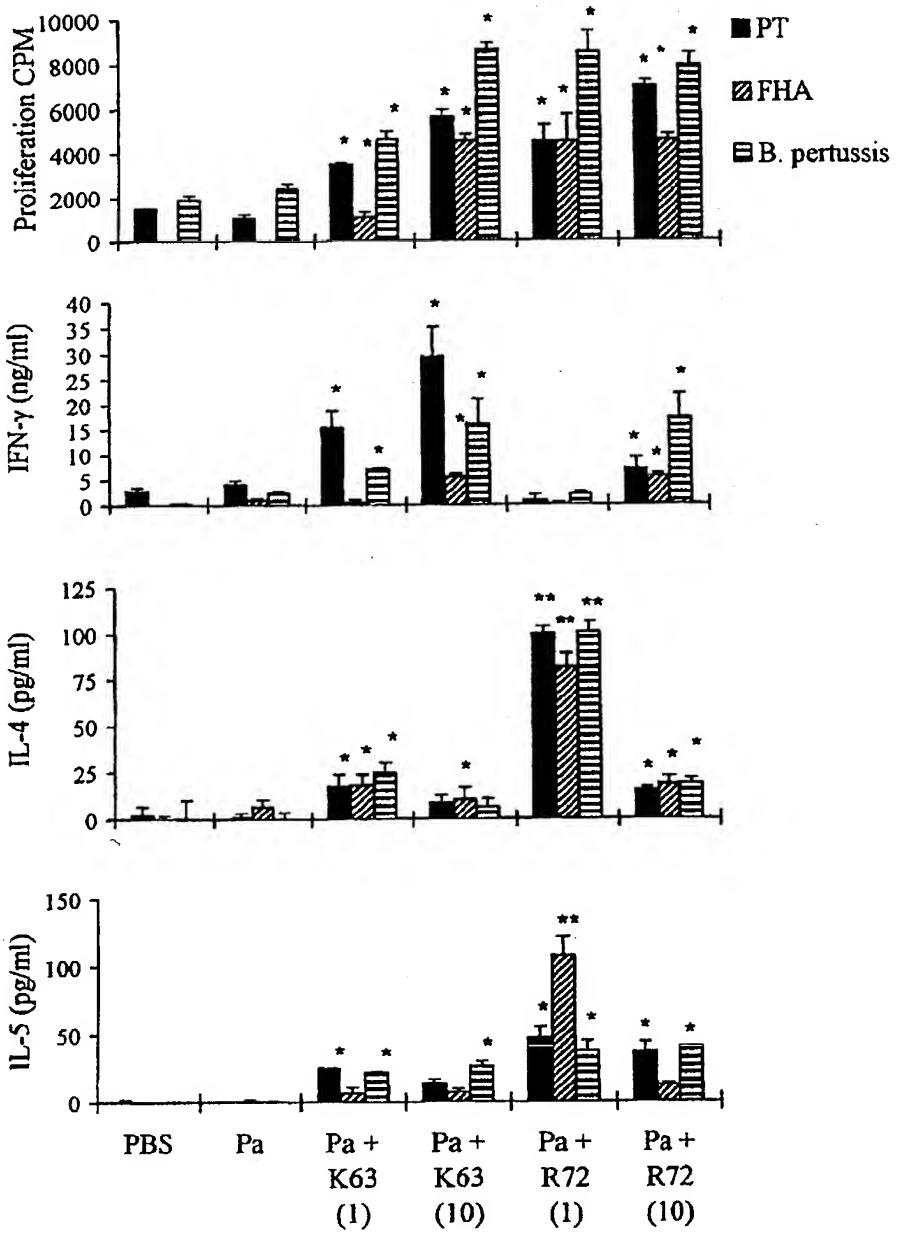


FIGURE 5A

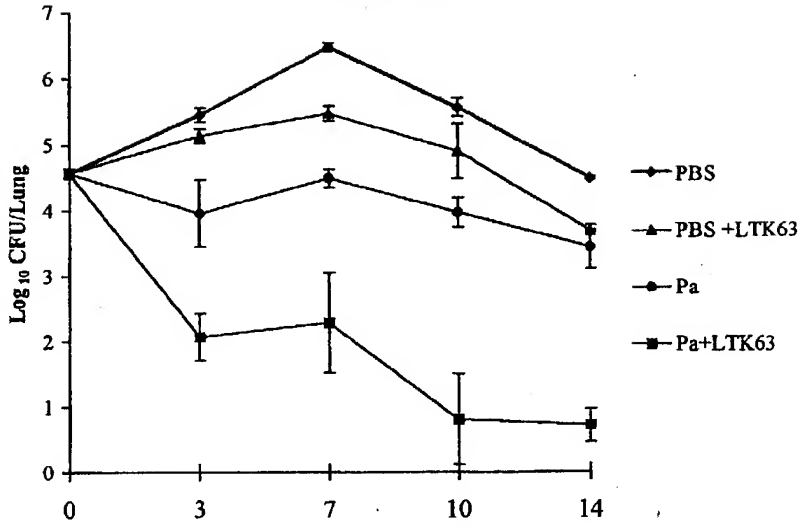


FIGURE 5B

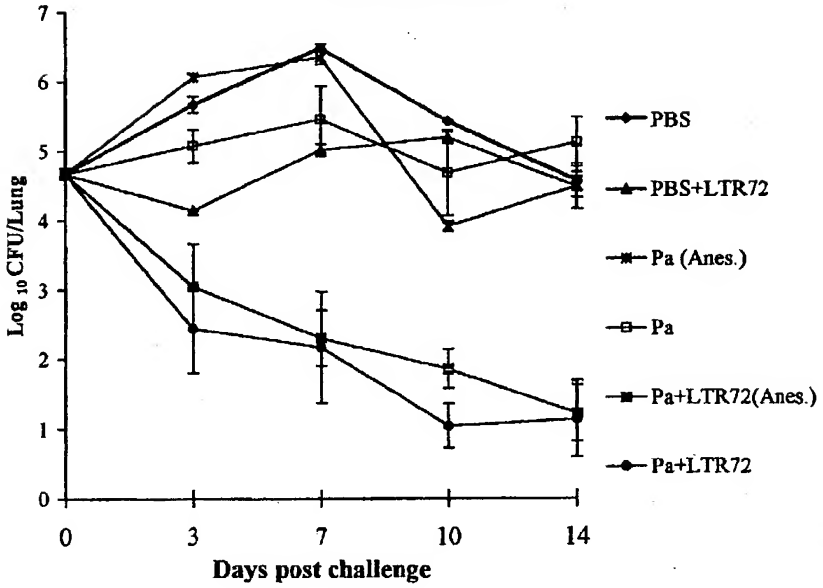


FIGURE 6

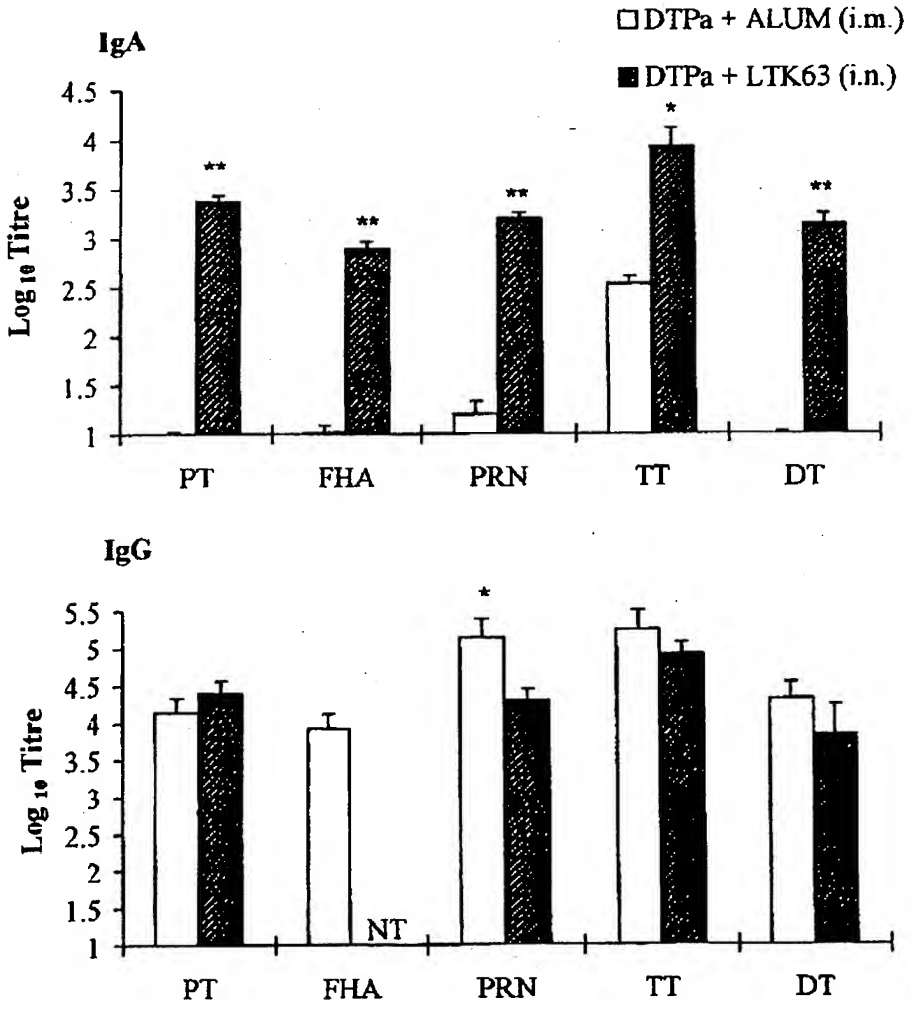

DTPa + ALUM (i.m.)
DTPa + LTK63 (i.n.)
IgA
Log 10 Titre
4.5
4
3.5
3
2.5
2
1.5
1
**
**
**
*
**
PT
FHA
PRN
TT
DT
IgG
Log 10 Titre
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
*
NT
PT
FHA
PRN
TT
DT

FIGURE 7

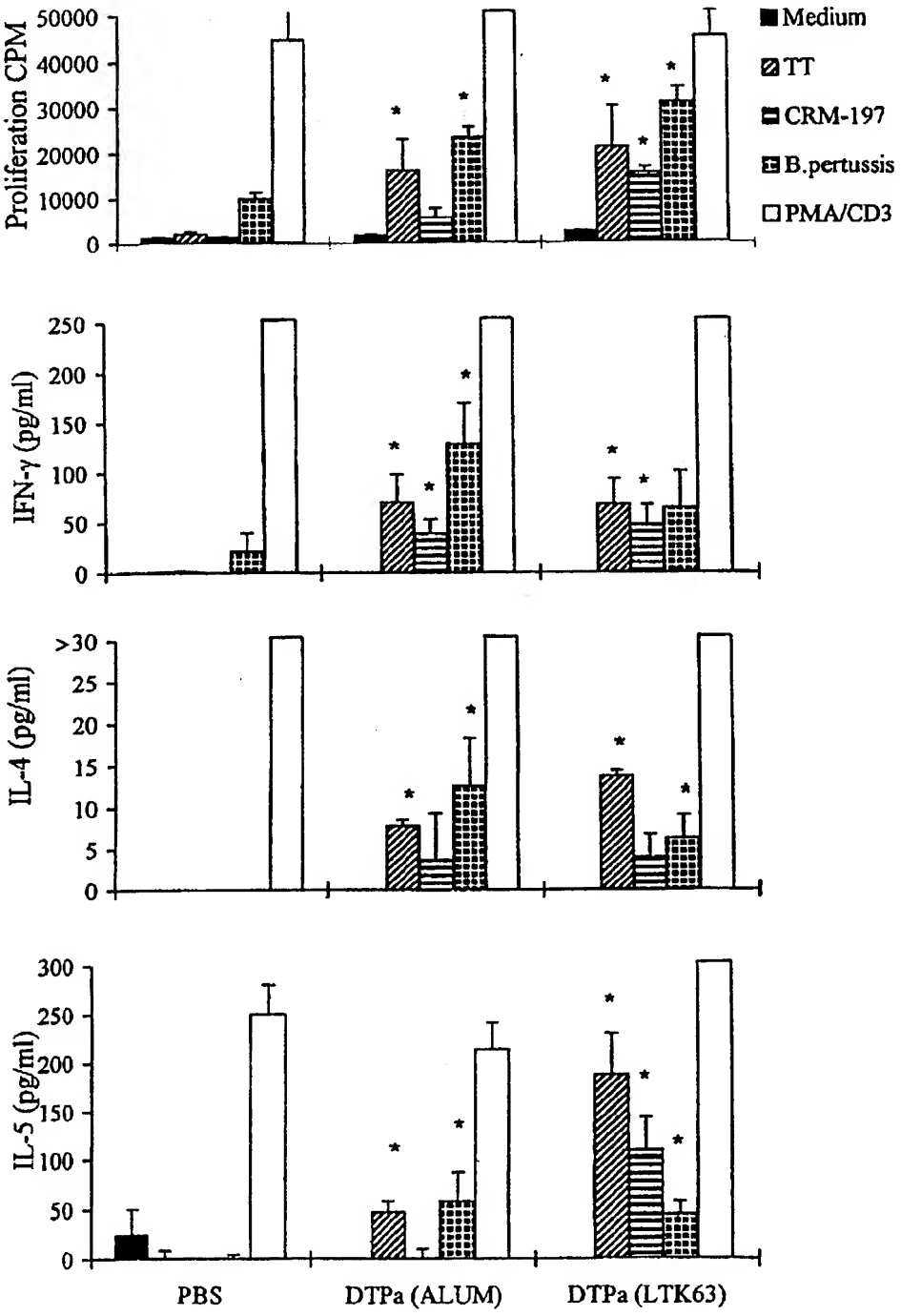

Proliferation CPM
50000
40000
30000
20000
10000
0
Medium
TT
CRM-197
B.pertussis
PMA/CD3
IFN-γ (pg/ml)
250
200
150
100
50
0
IL-4 (pg/ml)
>30
25
20
15
10
5
0
IL-5 (pg/ml)
300
250
200
150
100
50
0
PBS
DTPa (ALUM)
DTPa (LTK63)

FIGURE 8

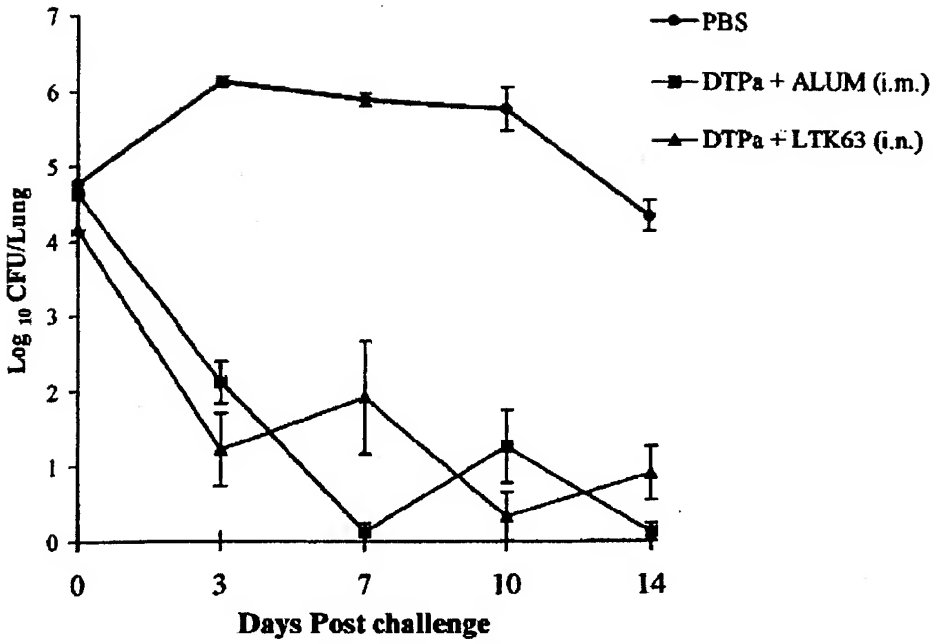
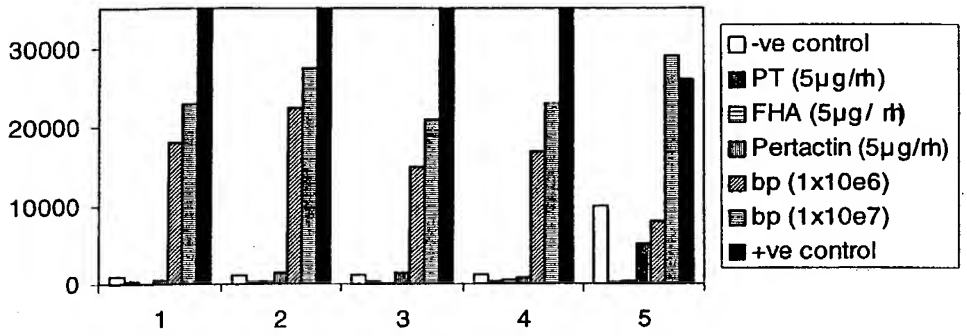
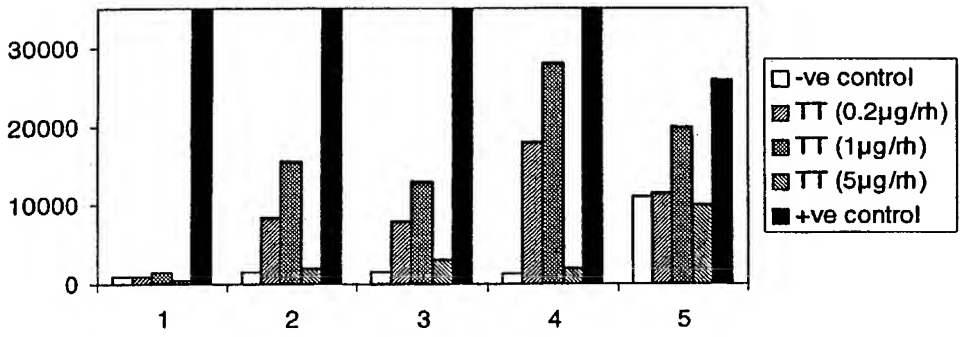

PBS
DTPa + ALUM (i.m.)
DTPa + LTK63 (i.n.)
Log 10 CFU/Lung
0
1
2
3
4
5
6
7
0
3
7
10
14
Days Post challenge

FIGURE 9


30000
20000
10000
0
1
2
3
4
5
-ve control
PT (5µg/ml)
FHA (5µg/ ml)
Pertactin (5µg/ml)
bp (1x10e6)
bp (1x10e7)
+ve control


30000
20000
10000
0
1
2
3
4
5
-ve control
TT (0.2µg/ml)
TT (1µg/ml)
TT (5µg/ml)
+ve control

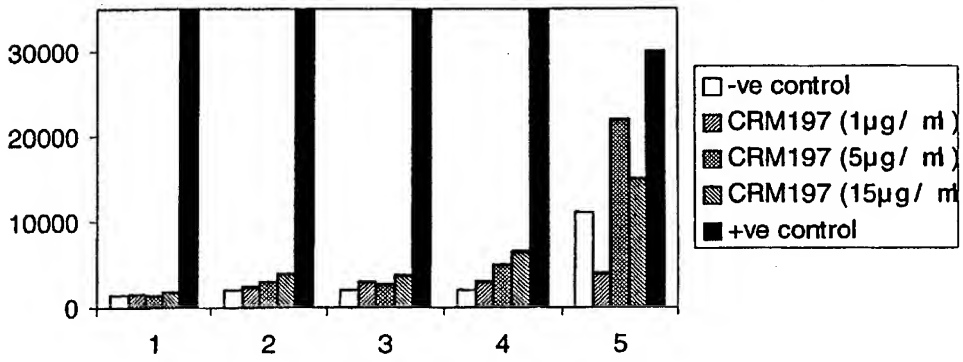

30000
20000
10000
0
1
2
3
4
5
-ve control
CRM197 (1µg/ ml)
CRM197 (5µg/ ml)
CRM197 (15µg/ ml
+ve control

FIGURE 10

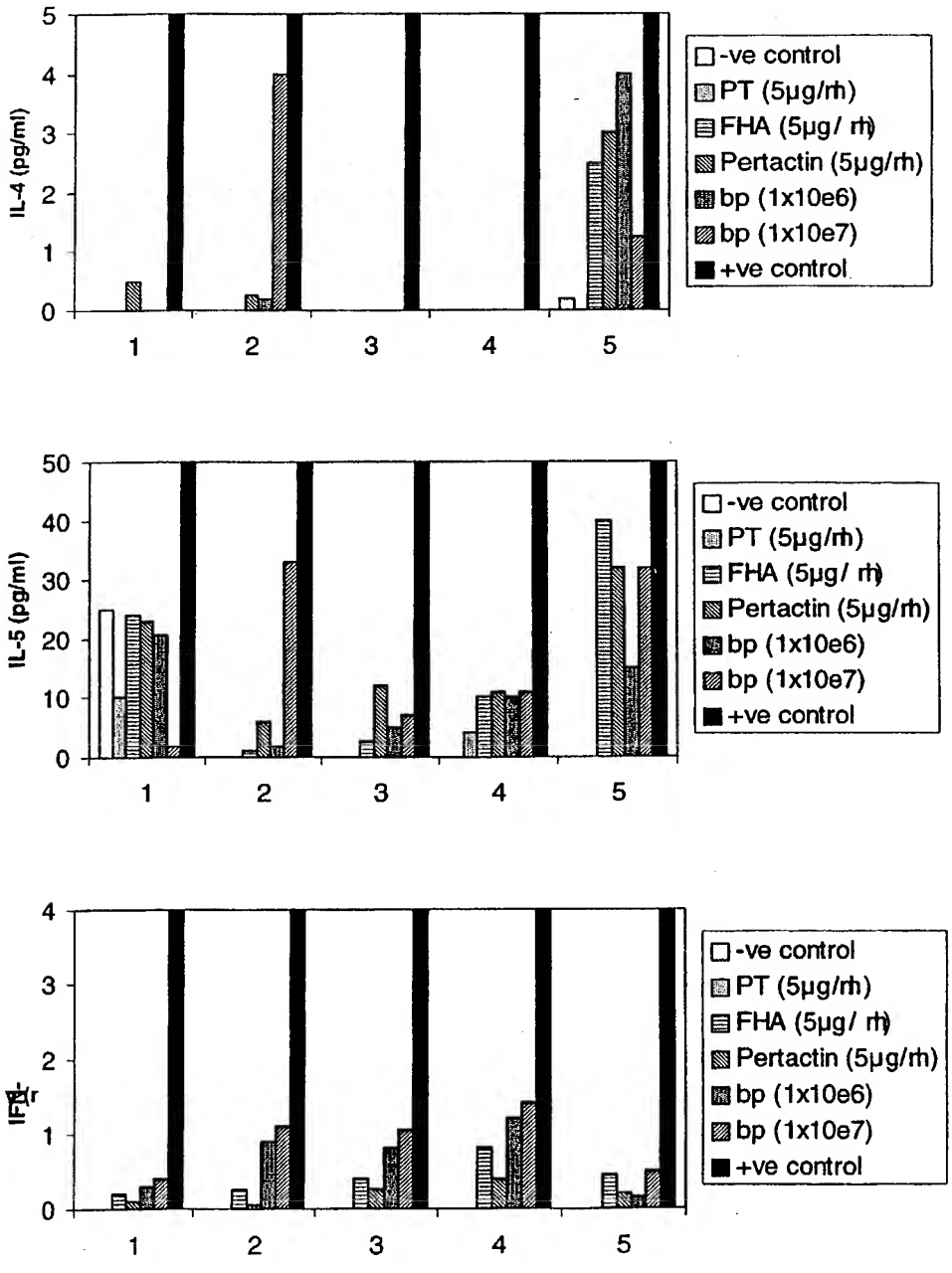

IL-4 (pg/ml)
0
1
2
3
4
5
1
2
3
4
5
-ve control
PT (5µg/rh)
FHA (5µg/ rh)
Pertactin (5µg/rh)
bp (1x10e6)
bp (1x10e7)
+ve control
IL-5 (pg/ml)
0
10
20
30
40
50
1
2
3
4
5
-ve control
PT (5µg/rh)
FHA (5µg/ rh)
Pertactin (5µg/rh)
bp (1x10e6)
bp (1x10e7)
+ve control
IFN-γ
0
1
2
3
4
1
2
3
4
5
-ve control
PT (5µg/rh)
FHA (5µg/ rh)
Pertactin (5µg/rh)
bp (1x10e6)
bp (1x10e7)
+ve control

FIGURE 11

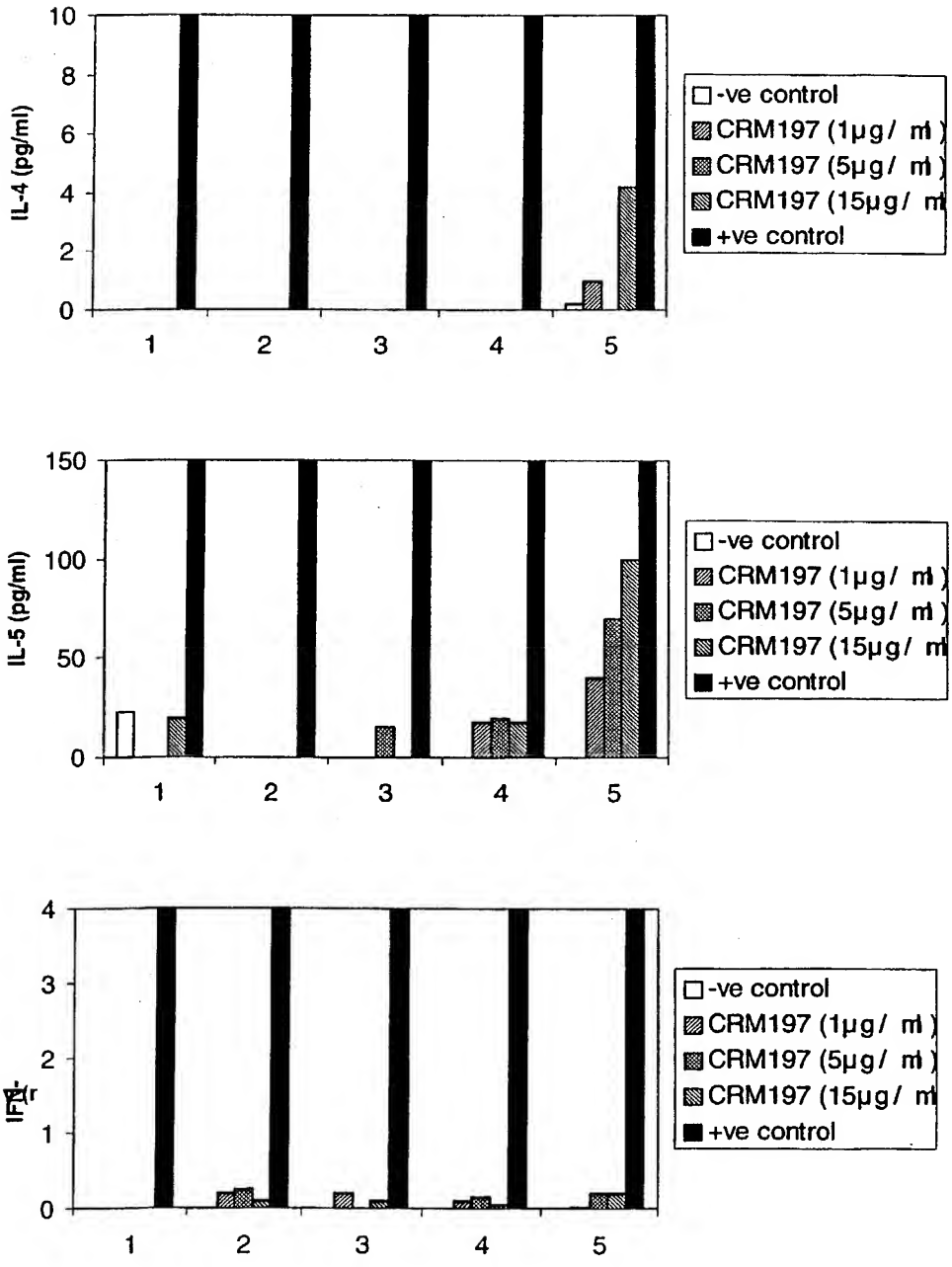
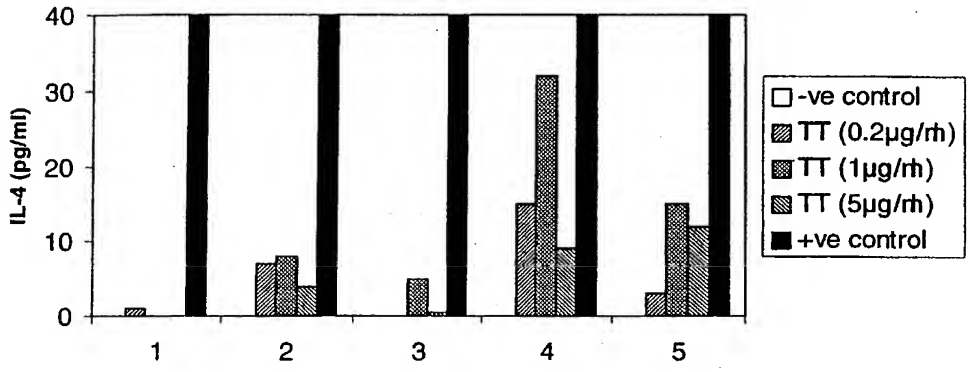
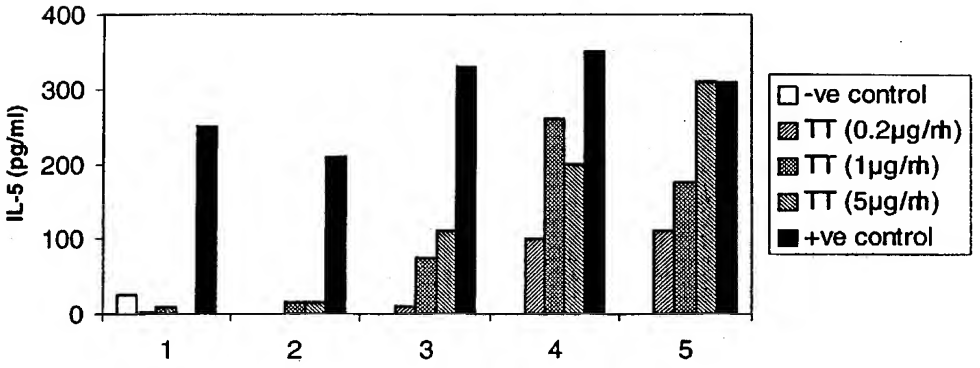

IL-4 (pg/ml)
10
8
6
4
2
0
1
2
3
4
5
-ve control
CRM197 (1µg/ ml)
CRM197 (5µg/ ml)
CRM197 (15µg/ ml)
+ve control
IL-5 (pg/ml)
150
100
50
0
1
2
3
4
5
-ve control
CRM197 (1µg/ ml)
CRM197 (5µg/ ml)
CRM197 (15µg/ ml)
+ve control
4
3
2
1
0
1
2
3
4
5
-ve control
CRM197 (1µg/ ml)
CRM197 (5µg/ ml)
CRM197 (15µg/ ml)
+ve control

FIGURE 12


40
30
20
10
0
IL-4 (pg/ml)
1
2
3
4
5
-ve control
TT (0.2µg/rh)
TT (1µg/rh)
TT (5µg/rh)
+ve control


400
300
200
100
0
IL-5 (pg/ml)
1
2
3
4
5
-ve control
TT (0.2µg/rh)
TT (1µg/rh)
TT (5µg/rh)
+ve control

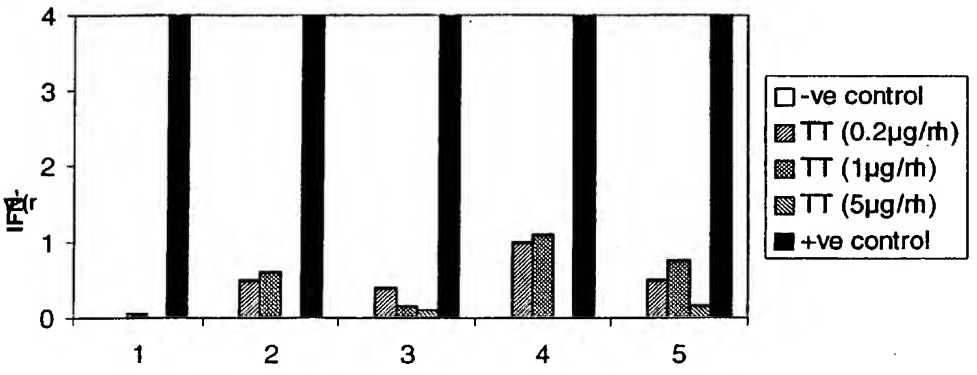

4
3
2
1
0
1
2
3
4
5
-ve control
TT (0.2µg/rh)
TT (1µg/rh)
TT (5µg/rh)
+ve control

FIGURE 13

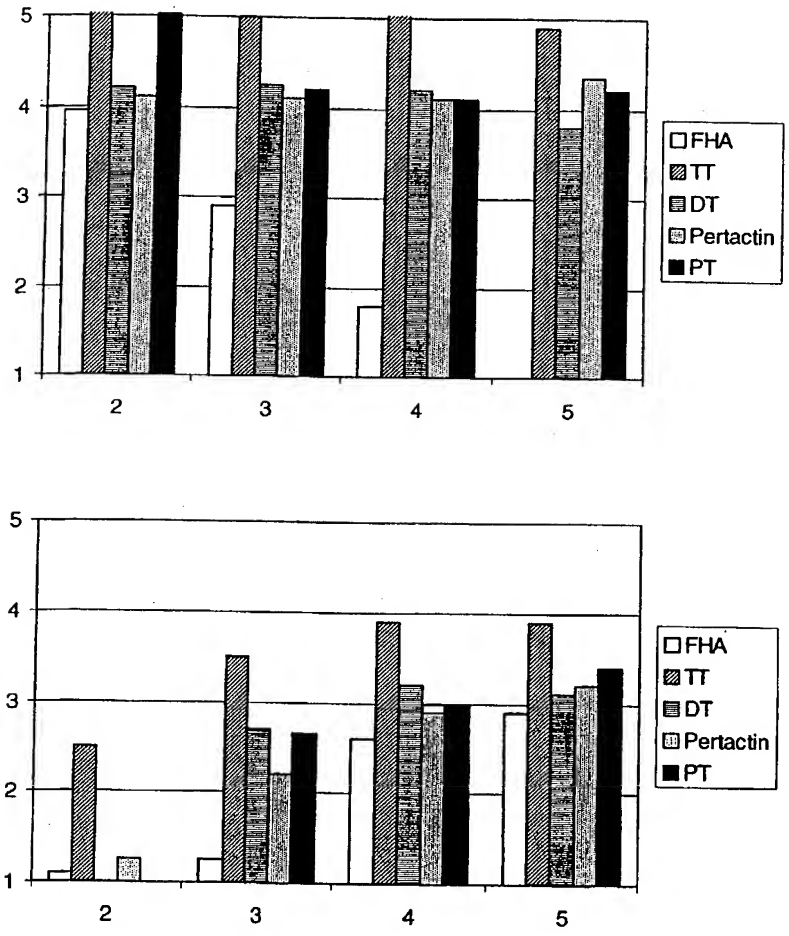

5
4
3
2
1
2
3
4
5
FHA
TT
DT
Pertactin
PT
5
4
3
2
1
2
3
4
5
FHA
TT
DT
Pertactin
PT

FIGURE 14

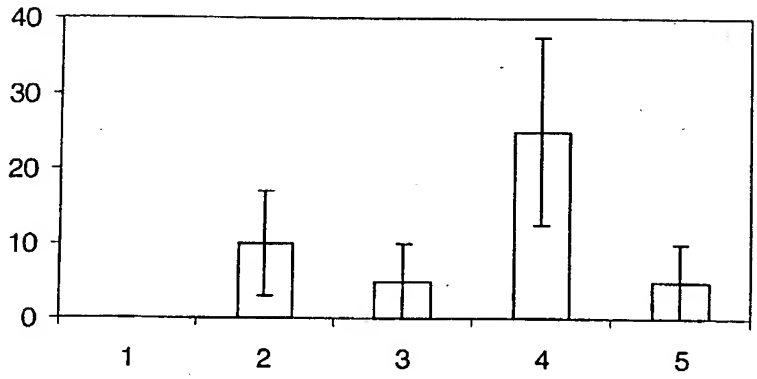
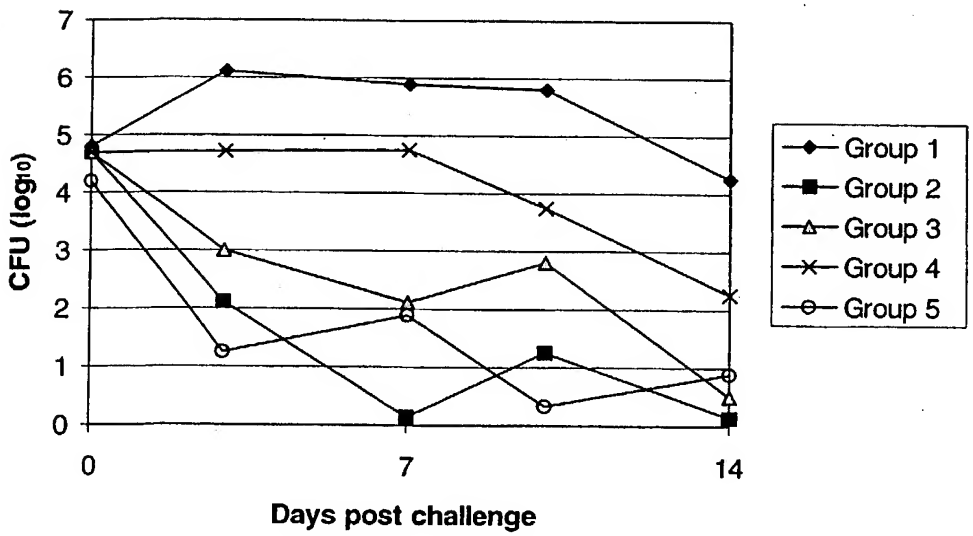


FIGURE 15



PTO/SB/01 (10-01)
Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing OR ☑ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	1651.102
First Named Inventor	Rappuoli, Rino
COMPLETE IF KNOWN	
Application Number	10/089,367
Filing Date	
Art Unit	
Examiner Name	

As the below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original and first inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MUCOSAL DTPa VACCINES

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☑ was filed on (MM/DD/YYYY) 09/28/2000 as United States Application Number or PCT International Application Number PCT/IB00/01440 and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
9923060.9	GB	09/29/1999	☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

PTO/SB/01 (10-01)
Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION — Utility or Design Patent Application

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NAME OF SOLE OR FIRST INVENTOR : ☐ A petition has been filed for this unsigned inventor

100

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☐ Additional inventors are being named on the ____supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.